Pathogens of Medically Important Mosquitoes of Thailand

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OBJECTIVE : To determine the kinds of mosquito pathogens occurring in medically important mosquitoes in Thailand and to elucidate the biology of selected pathogens sufficiently to assess their potential as biological mosquito control agents.

BACKGROUND : Results of surveys to determine the kinds and distributions of mosquito pathogens infecting *Aedes aegypti* and *Culex pipiens quinquefasciatus* in Thailand were previously reported (1, 2). Among the 16 host-pathogen systems found in these species, 13 were considered worthy of evaluation as potential biological control agents; i.e., the pathogens represented taxa containing organisms known to be pathogenic to mosquitoes. Unfortunately, some of these systems were rare or were found far from the laboratory. Evaluation of biological control potential was initiated with six accessible systems. These represented what appeared to be four species of pathogens, two of which occurred in both *A. aegypti* and *C. pipiens quinquefasciatus*. Microsporidan #1, probably *Stempellia milleri*, was found in *C. pipiens quinquefasciatus*. Microsporidan #2, possibly also of Genus *Stempellia*, since pansporoblasts with both four and eight sporoblasts occur in its developmental cycle, occurred in *A. aegypti*. An helicosporidian, near *Helicosporidium parasiticum*, occurred in both *A. aegypti* and *C. pipiens quinquefasciatus* throughout Thailand. A minute, and as yet unculturable and unidentified, gram negative, motile, vibrioform bacterium also occurred in both host species and has been observed to be the probable cause of massive epizootics in larval *C. pipiens quinquefasciatus*.

The general approach to evaluating biological control potential in the laboratory consisted of, first, establishing the pathogens in the laboratory and, then, conducting experiments with them to determine the degree to which they possessed characteristics desired in biological control agents for mosquitoes. Since no pathogen has yet been developed for large scale use against mosquitoes, desired "standards of performance" were hypothetical. A biological agent to be used against mosquitoes should possess at least the following characteristics : (1) it should kill mosquitoes or otherwise interfere with their ability to transmit diseases, (2) its effects on non-target organisms should be ecologically acceptable, (3) the cost of its production and use should be acceptable, (4) it should have a shelf-life of acceptable duration, and (5) it should not be neutralized by the environment into which it is dispersed until it has infected the desired proportion of the target population.

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METHODS : Methods commonly used will be described here. Those specific to certain experiments will be described where appropriate. Mosquito larvae infected with the pathogens of immediate concern displayed gross signs of pathology which permitted the identification and collection of heavily infected larvae in the field. These were transported to the laboratory and used as a source of inoculum for the establishment of pathogens in laboratory reared mosquitoes. Once this was accomplished, laboratory produced pathogens were used as inocula for experiments.

The stock mosquito colony was maintained in a separate insectary into which pathogens were never taken, using equipment kept only in that insectary. Pathogen suspensions for use as inoculum were prepared by triturating infected hosts in a Ten-Broeck type tissue grinder until the head capsules were destroyed. The triturant was filtered through organdy cloth to remove large debris and washed by centrifugation. Protozoan pathogens (helicosporida and microsporida) were quantified using a hemocytometer. Bacterial inoculum was quantified crudely in units of larval equivalents, one larval equivalent being the bacteria from one patently infected larva. Per os exposures of uninfected larvae were made in 85 mm plastic petri dishes that were washed and ethanol sterilized between usages. Routinely, 200 mosquito larvae were placed in these with 20 ml of pathogen suspension. A small quantity of food was provided during exposure. Except in experiments where duration of exposure was a variable, exposures were of 24 hr duration. Following exposure, the larvae were poured onto an organdy cloth screen, gently rinsed with tap water, and then placed in rearing pans. The contents of one exposure container, 200 larvae, were placed in one pan. Post exposure rearing was in enamel or aluminum pans that were sterilized by autoclaving after use. Larvae were fed laboratory animal chow ground finely enough to pass through a 60 mesh screen.

Specimens for examination were usually collected from among the exposed larvae when pupae first appeared. It was advantageous to allow infections to develop as long as possible to facilitate their detection. However, many mosquito pathogens kill their hosts in the late fourth stage or during pupation, and this mortality makes it difficult to collect a random sample representing the exposed population.

Infections were detected by smearing mosquitoes onto microscope slides with the tips of wooden applicator sticks, five discrete smears per slide. The smears were air-dried, fixed for one minute with absolute methanol, and stained with Giemsa stain in 0.01 M pH 7.6 Tris buffer. Coverslips were applied, and the smears were examined at 500χ or 1250χ magnification. No less than 50 individuals from any experimental group were examined, except where indicated. Dose-response data at several dosages were commonly collected. These data were plotted on similog graph paper, regression lines were eye-fitted, and IC_{50}'s were estimated from these. The stock colony was routinely sampled to assure that it was free from pathogens.

Establishment of Pathogens in the Laboratory

Microsporidan #1 in *Culex pipiens quinquefasciatus*

METHODS : Field collected *Culex pipiens quinquefasciatus* had 1.7×10^6 spores per infected larva. Twenty-four hour old laboratory produced *C. pipiens quinquefasciatus* larvae were exposed to 3.4×10^6 spores per ml.

RESULTS : Seventy-seven percent transmission occurred among 100 specimens examined. Other transmission attempts using fewer spores or older larvae were unsuccessful. This pathogen was not routinely maintained in the laboratory, but it appears very likely that it could be.

Microsporidan #2 in *Aedes aegypti*

METHODS : *Aedes aegypti* larvae from several localities had from 2.5×10^5 to 3.8×10^5 (mean = 3.04×10^5) spores per infected larva. Twenty four hour old laboratory reared *A. aegypti* larvae were exposed.

RESULTS : Dosages of 4×10^3, 8×10^3, 2×10^4, 4×10^4 and 2×10^5 spores per ml produced 7, 16, 30, 84 and 100 percent infection, respectively, with an IC_{50} of between 3×10^4 and 4×10^4 spores per ml. This pathogen was routinely maintained in the laboratory with ease.

Microsporidan #2 in *Culex pipiens quinquefasciatus*

METHODS : Forty-eight hour old *Culex pipiens quinquefasciatus* larvae were exposed to 1×10^6 spores per ml.

RESULTS : No transmission was detected.

Helicosporidium sp. in *Culex pipiens quinquefasciatus*

METHODS : Field collected *Culex pipiens quinquefasciatus* larvae infected with this pathogen averaged 2.25×10^6 spores per larva. Early second stage larvae were exposed.

RESULTS : Dosage of 1×10^4, 2×10^4, 5×10^4, 1×10^5 and 2.5×10^5 spores per ml produced 8, 32, 76, 84 and 100 percent infection, respectively, with an IC_{50} of about 1.35×10^5 spores per ml.

Helicosporidium sp. in *Aedes aegypti*

METHODS : *Aedes aegypti* larvae infected with this pathogen display much less conspicuous signs of infection than do infected *Culex pipiens quinquefasciatus*. Therefore, field collected *Culex* were initially used as a source of inoculum to establish this pathogen in *A. aegypti* by exposing 48 hr old larvae to various dosages.

RESULTS : Difficulty was experienced establishing this pathogen in *A. aegypti*. Dosage routinely used to infect *Culex* caused massive mortality. Eventually, by exposing *A. aegypti* to much lower dosages, a highly efficient system for the production of this pathogen was developed. Exposure of 48 hr old larvae to dosages of 5×10^2, 1×10^3, 5×10^3, and 1×10^4 spores per ml produced 8, 24, 90 and 100 percent infection, respectively, with an IC_{50} of about 1.9×10^3.

Helicosporidium sp. in *Anopheles balabacensis*

METHODS : Both second and third stage *Anopheles balabacensis* were exposed to *Helicosporidium* spores produced in the laboratory in *Aedes aegypti*.

RESULTS : Exposure of second stage *An. balabacensis* larvae to dosages of 1×10^3, 5×10^3, 1×10^4 and 5×10^4 spores per ml produced 60, 96, 100 and 100 percent infection, respectively, with an IC_{50} of less than 1×10^3 spores per ml. Exposure of third stage larvae to dosages 1×10^3, 5×10^3, 1×10^4, 5×10^4, 1×10^5, 5×10^5 and 1×10^6 spores per ml produced 8, 16, 20, 56, 88, 100 and 100 percent infection, respectively with an IC_{50} of about 2.5×10^4. The sharp increase in the IC_{50}'s with age of larvae at exposure was commonly encountered.

Helicosporidium sp. in *Anopheles maculatus*

METHODS : *Anopheles maculatus* from Malaysia, where the species is an important vector of human malaria, were exposed as second stage larvae to *Helicosporidium* spores produced in *Aedes aegypti*.

RESULTS : Exposure of larvae to dosages of 7.5×10^3, 11.25×10^3, and 1.5×10^4 spores per ml produced 10, 22 and 48 percent infection, respectively, with a probable IC_{50} of about 1.7×10^4 spores per ml.

Unidentified Bacterium in *Culex pipiens quinquefasciatus*

METHODS : Late second stage *Culex pipiens quinquefasciatus* larvae were exposed to 0.75 larval equivalents per ml of this agent alone and along with 5×10^3 *Helicosporidium* spores per ml.

RESULTS : No transmission of the bacterium occurred to larvae exposed to it alone. In the groups exposed to both the bacterium and to the protozoan, mortality was very high in early larval stages. Of those surviving to the fourth stage, 12 percent were infected with *Helicosporidium* alone, 8 percent were infected with the bacterium alone and 2 percent were infected with both pathogens. Because of the high mortality occurring soon after exposure, these results probably are deceptive. In experiments described below, larval mortality occurring soon after exposure appeared to be due to septicemia, and a possible mechanism is discussed.

Experiments Pertinent to Evaluation of Biological Control Potential

Effects of Age of *Aedes aegypti* Larvae at Exposure on Percent Transmission in Microsporidan #2

METHODS : *A. aegypti* larvae were exposed to 5×10^4 spores per ml of Microsporidan #2 during the second, third, fourth and fifth days of larval life. These ages corresponded to the second, early third, early fourth, and late fourth larval stages, respectively. An additional group was exposed to 1×10^5 spores per ml during the third day of life, when the larvae were in the early third stage.

RESULTS : Transmission rates of 100, 2, 0 and 0 percent, respectively, were achieved with the first four groups, indicating a precipitous decrease in susceptibility with increasing age. One-hundred percent transmission was achieved in the group exposed to 1×10^5 spores per ml, indicating that an increase in dosage could compensate for the decrease in susceptibility, at least in larvae of the age tested.

Vertical Transmission of Microsporidan #2 in *Aedes aegypti*

METHODS : *Aedes aegypti* larvae, 24 hrs old, were exposed to 7.5×10^4 spores per ml. When pupation began, smears were made of a random sample of fourth stage larvae, and percentage transmission was determined. Pupae were collected and surviving adults were given a blood meal and allowed to oviposit. Samples of the ova were hatched after one week and 10 weeks storage at room temperature. Resulting larvae were reared to advanced fourth stage, and the percent infected was determined. Adult survivors of the larvae hatched after one week storage were allowed to oviposit, and these progeny and their progeny were checked for infection with the microsporidan.

RESULTS : Infection occurred in 95 percent of the exposed larvae. The progeny of the exposed generation hatched from ova stored for only one week were 45 percent infected. After storage for an additional nine weeks, the percent infection in progeny was reduced to 4 percent, suggesting that ova carrying the infection died more rapidly than those which did not. No transmission was

detected in generations after the immediate progeny of the exposed generation.

Effects of Age of *Aedes aegypti* larvae at Exposure on Percent Transmission of *Helicosporidium* sp.

METHODS : Groups of *Aedes aegypti* larvae of increasing ages were exposed to the dosages of *Helicosporidium* spores indicated in Table 1. When population began, 100 larvae from each experimental group were examined to determine percent infection. IC_{50}'s were estimated from dose-response data. Infections in the remaining larvae were allowed to develop until the larvae died or pupated, at which time they were held at 4°C. When all larvae had died or pupated, a random sample of 100 from each age group were triturated, and the number of spores produced per infected individual was determined.

RESULTS : (See Table 1.) IC_{50} was lowest for larvae exposed for 24 hrs beginning at 48 hrs of age. This was unexpected and will be discussed briefly below. The number of spores produced per infected larva was inversely proportional to age of larvae at exposure, reflecting the greater time available for development of infections in larvae that were younger when exposed. It is also possible that younger larvae were less resistant to penetration of the infectious agent, and, therefore, received a heavier initial dose.

Mortality was not quantified in this experiment, but it was noticed to be bimodal. One peak occurred soon after exposure and another in the late fourth larval stage. Mortality in the first peak seemed to be heaviest in younger larvae and in larvae exposed to heavier dosages. Developing *Helicosporidium* was seldom found in these larvae. All were septicemic. Possibly, bacteria penetrated the gut wall along with the sporozoites of *Helicosporidium* and multiplied in the hemolymph rapidly enough to kill the larvae before the protozoan infection developed. The unexpectedly high IC_{50}'s in young larvae might have resulted from high mortality occurring soon after exposure among younger infected larvae. Differential mortality in these groups would distort dose-response data in such a way as to lower IC_{50}'s derived from them.

Forty-eight hour old larvae were considered ideal for dose-response experimentation. They were strong enough to survive exposure, yet, enough time remained in the larval stage for most infections to be well developed before the second mortality peak began decimating the exposed populations.

Effects of Duration of Exposure of *Aedes aegypti* Larvae on Percent Transmission of *Helicosporidium* sp.

METHODS : Groups of *Aedes aegypti*larvae, 48 hrs old, were given exposures of increasing duration to a range of dosages of *Helicosporidium* spores. When the exposures at each duration were completed, the larvae were placed on organdy cloth screens and rinsed gently with tap water, before returning them to a clean exposure container until the exposure of maximum duration was completed.

Following exposure, rearing procedures described above were followed, and random samples to determine percent transmission were taken of the exposed larvae when pupation began. IC_{50}'s were estimated from dose-response data.

RESULTS : As indicated in Table 2, an inverse relationship existed between duration of exposure and percent transmission. Brief exposures produced 100 percent transmission only if dosage was high. Twenty-four hour exposures were adopted as routine, because exposures of that duration required only about one-half as much inoculum to effect 100 percent transmission as required by one hour exposures. Larval mortality, though not quantified, seemed to increase with both duration of exposure and with dosage. If minimum dosages required to produce 100 percent infection in larvae of the age being used were administered, mortality was not a serious problem until infections were well developed.

Relationship Between Intensity of Exposure of *Aedes aegypti* to *Helicosporidium* Spores and Percent Infection, Mortality and Time of Occurrence of Mortality

METHODS : Three groups of 2400, 48 hr old *Aedes aegypti* larvae were exposed for 24 hrs to three different dosages of *Helicosporidium* spores, 1×10^4, 3×10^4 and 9×10^4. One dose was intended to give less than 100 percent infection, one dose was intended to give 100 percent infection without overwhelming the host; and one dose was intended to grossly overwhelm the host. Daily mortality data were recorded, until the mosquitoes were 15 days old, to determine the relationship between dose and mortality and time of occurrence of mortality. Beginning with mosquitoes that died between 24 and 48 hrs after the initiation of exposure the percentage of infection detectable in dead mosquitoes was determined at 24 hr intervals until the mosquitoes were 11 days old. Beginning 48 hrs after the initiation of exposure, daily samples of live larvae, larvae and pupae, pupae, pupae and adults, or adults (whichever forms were prevalent at the time of sampling) were examined to determine the percentage of infection in the proportion of the population still alive, until the mosquitoes were 11 days old. Controls were exposed to a triturant of uninfected larvae and reared in parallel with the experimental groups.

RESULTS : Only 4 percent mortality and no infection occurred in the control group during the two weeks of the experiment. This mortality was distributed throughout the development cycle of the mosquitoes, with 38.6 percent of total mortality occurring among larvae, 38.6 percent among pupae, and 22.8 percent among adults. The control data indicated that conditions of exposure and rearing were not unduly harmful to the mosquitoes.

Mortality among the group exposed to 1×10^4 spores per ml was 62.3 percent. Of that mortality, 27.4 percent occurred among larvae, 57.4 percent among pupae, and 15.2 percent among adults. Mortality among the group exposed to 3×10^4 spores per ml was 92.8 percent. Of that mortality, 36.7 percent occurred among larvae, 54.5 percent among pupae, and 8.8 percent among adults. Mortality among mosquitoes exposed to 9×10^4 spores per ml was 98.0 percent. Of that mortality, 79.5 percent occurred among larvae, 18.0 percent among pupae, and 2.4 percent

among adults. Daily occurrence of mortality is shown in Table 3, and the bimodality of mortality is evident. An increasing proportion of total mortality occurred soon after exposure as dosage increased. Also, the second peak of mortality occurred earlier as dosage increased, indicating that time of mortality was related to intensity of exposure. This was reflected in the distribution of mortality between larvae, pupae and adults in the three experimental groups.

Actual percentages of infection resulting from exposure to *Helicosporidium* were difficult to determine. Difficulties resulting from mortality soon after exposure were mentioned above. As illustrated in Table 4, percentage of infection that could be detected varied with time since exposure and with mortality that had occurred prior to collecting samples for examination. Among larvae exposed to relatively light doses, not only did less transmission occur, but infections were more difficult to detect soon after exposure. A smaller proportion of total infections were found to occur, detected 48 or 72 hrs after initiation of exposure, in lightly exposed groups than in heavily exposed groups Also deceptive were percents of infection taken after the second peak of mortality. Only 60 percent infection was detected in adults surviving for nine days after initiation of the heaviest exposure. Samples taken as early as the fourth day after initiation of exposure in that group were 98 percent infected. Most of the exposed population (95.8 percent) died before the ninth day after initiation of infection, and the few uninfected individuals became a conspicuous proportion of the remaining population.

The most accurate single determination of percent transmission could be made from samples collected just before the occurrence of the second mortality peak. By that time, most infections became detectable by the methods used, and distortions of dose-response data caused by the second mortality peak were not yet a problem. In all three exposure groups, the second mortality peak began on day six after the initiation of exposure, so dose-response data from day five should be the most accurate. The peak in mortality rate occurred one day earlier for each progression in dosage.

Retention of Infectivity by *Helicosporidium* Spores Stored in Demineralized Water at 4°C

METHODS : Spores were recovered from infected *Aedes aegypti* larvae and washed three times by centrifugation at 750 x g for 20 min at 4°C. The spores were divided into aliquots and stored in a refrigerator in demineralized water at 4°C in cotton-stoppered glass test tubes. Baseline dose-response data was acquired with a portion of the spores before storage. After one and two weeks of storage and biweekly thereafter until 16 weeks of storage, aliquots of the spores were evaluated for infectivity.

RESULTS : As indicated in Table 5, considerable loss of infectivity occurred during the 16 wk storage period, but storage at 4°C for short periods should be useful to investigators. Spore counts per unit volume remained essentially the same throughout the storage period, indicating no degeneration of physical integrity of the spores.

Retention of Infectivity by *Helicosporidium* Spores at Room Temperature in Buffer Solution and in Buffered Solutions of Antibiotics

METHODS : Spores were recovered from infected *Aedes aegypti* larvae and washed three times by centrifugation at 750 x g for 20 min at 4°C in phosphate buffered saline, pH 7.0. After the final wash, aliquots of the spores were resuspended in the appropriate storage medium. Four storage media were tested : (1) 0.05 M phosphate buffer, pH 7.0, (2) 400 units of Potassium Penicillin G and 1.0 mg Streptomycin Sulfate per ml of the same buffer, (3) 1000 units of Polymyxin B Sulfate per ml of buffer, and (4) 1.5 mg Kanamycin Sulfate per ml of buffer. Baseline dose-response data were acquired with untreated spores. Suspensions of the treated spores were stored in the dark at 25°C, and infectivity was tested at intervals of 10, 17 and 24 days.

RESULTS : No transmission was acquired with spores stored in Penicillin-Streptomycin or in Kanamycin. As indicated in Table 6, some infectivity was retained by spores stored in buffer and in buffered Polymyxin B for up to 17 days. No transmission occurred with spores stored for 24 days.

Retention of Infectivity by *Helicosporidium* Spores Stored by Lyophilization, Vacuum Drying and Freezing*

METHODS : Spores of *Helicosporidium* were preserved in intact, infected *Aedes aegypti* larvae by freezing in liquid nitrogen (-196°C) and were then stored in liquid nitrogen and in a REVCO freezer at -70°C. Spores in intact larvae were also preserved by lyophilization and by vacuum drying and stored at -70°C and at room temperature. Spores recovered from infected larvae by trituration and centrifugation were preserved by freezing in liquid nitrogen as suspensions in two cryo-protectants, one based on dimethyl sulfoxide (3) and one based on egg yolk and glycerine (4). These were stored in liquid nitrogen and at -70°C. Baseline dose-response data was acquired with fresh spores. At intervals of 4, 8, 12 and 16 weeks, infectivity of the spores was evaluated and dose-response data acquired. This data was used to eye-fit curves of the regression of dosage on percent transmission. IC_{50}'s were estimated from the regression lines.

RESULTS : Some transmission was acquired after four weeks with spores subjected to all preservation and storage methods. However, methods employing drying permitted very poor retention of infectivity. The IC_{50}'s of spores preserved by lyophilization or vacuum drying increased by more than a factor of 100 (2 logs). Storage and preservation by all methods involving freezing without drying were highly effective, as shown in Table 7.

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* This project constituted the research for a Master of Science Degree for Miss Boongeua Witethom, Department of Biology, Chulalongkorn University, Bangkok, Thailand.

Many of the IC_{50}'s for stored material were lower than the IC_{50}'s for fresh spores. All IC_{50}'s for the 16th week of storage were less than those after only four weeks of storage. The temperature in the insectaries in which the experimental larvae were reared was uncontrolled. The experiments were done during a season of generally decreasing ambient temperatures. All tests were initiated with 48 hr old larvae, but, as the temperature dropped or rose from week to week during the 48 hr pre-exposure incubation period, the larvae against which spore infectivity was tested were smaller or larger; i.e., their biological age was less or more. The sensitivity of percent transmission to age of larvae at exposure was shown above. It was hypothesized that temperature variation precipitated the aberrant results. These data illustrate the futility of attempting quantitative experimental pathology under uncontrolled conditions.

Preliminary Observations on the Infectivity of *Helicosporidium* Spores to the Golden Hamster

METHODS: Eleven adult male and one adult female golden hamsters were given 40×10^6 *Helicosporidium* spores in drinking water over a 12 hr period. For 30 days they were observed for weight loss and behavior changes. After 30 days, they were weighed and necropsied. Observations for gross pathology were made and samples of organs were preserved in neutral buffered formalin for histological examination (not yet done).

RESULTS: No weight loss or change in behavior was observed. The eleven males appeared normal when examined for gross pathology. Pyometra was observed in one uterine horn of the single female examined. Results of histological examinations will be reported when completed.

Infectivity of *Helicosporidium* sp. for *Toxorhynchites splendens*, a Mosquito Predator of Mosquitoes

METHODS: One-hundred first stage and 100 third stage *Toxorhynchites* larvae were exposed for 24 hrs to 1×10^6 *Helicosporidium* spores per ml. Following exposure, they were fed a liberal diet of uninfected *Aedes aegypti* larvae of appropriate size until they either died or pupated. Dead larvae and pupae were examined for presence of *Helicosporidium* as described above.

One-hundred third stage *Toxorhynchites* larvae were fed a diet of three known *Helicosporidium* infected fourth stage *A. aegypti* larvae for five successive days. Subsequent to exposure, the *Toxorhynchites* larvae were fed a diet of uninfected larvae until they either died or pupated. Dead larvae and pupae were smeared and examined for *Helicosporidium* infections as described above.

RESULTS: Third stage larvae were apparently refractory to infection by spore suspension. Twenty-five percent of first stage larva exposed to spore suspension became infected. Four percent of third stage larvae became infected, when they were allowed to feed on infected *A. aegypti* larvae. The spore concentration

required to infect 25 percent of first stage *Toxorhynchites* larvae was 116 times that required to infect 50 percent of 48 hr old *A. aegypti* larvae.

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Table 1. Effects of age of *Aedes aegypti* at exposure to *Helicosporidium* sp. on percent transmission and spore production.

Age at Exposure	Dose (Spores/ml)	Percent Transmission	Spores Produced Per Infected Larva
2 hrs	1×10^3	10	3.4×10^6
	5×10^3	18	3.0×10^6
	1×10^4	32	2.4×10^6
($IC_{50} = 2 \times 10^4$)	2×10^4	42	1.5×10^6
	3×10^4	68	1.1×10^6
24 hrs	1×10^3	12	3.6×10^6
	5×10^3	46	1.7×10^6
	1×10^4	92	1.3×10^6
($IC_{50} = 4 \times 10^3$)	2×10^4	100	1.3×10^6
	5×10^4	100	1.5×10^6
48 hrs	1×10^3	28	4.3×10^5
	5×10^3	70	6.0×10^5
	1×10^4	86	7.0×10^5
($IC_{50} = 1.9 \times 10^3$)	2×10^4	98	7.5×10^5
	5×10^4	100	9.5×10^5
72 hrs	5×10^3	8	1.3×10^5
	1×10^4	6	8.1×10^5
	3×10^4	18	5.3×10^4
($IC_{50} = 3 \times 10^5$)	5×10^4	20	2×10^4
	7×10^4	42	1.4×10^5

Table 2. Percent transmission of *Helicosporidium* to *Aedes aegypti* larvae following exposures of increasing duration.

Dose (Spores/ml)	Duration of Exposure (Hours)							
	1	4	8	16	24	32	40	48
5×10^2	4	4	6	6	8	10	10	12
1×10^3	6	10	14	20	24	28	32	36
5×10^3	46	72	80	88	90	95	100	100
1×10^4	94	100	100	100	100	100	100	100
5×10^4	100	100	100	100	100	100	100	100
IC_{50}'s	3.5×10^3	2.6×10^3	2.2×10^3	2.0×10^3	1.9×10^3	1.8×10^3	1.45×10^3	1.4×10^3

Table 3. Percentage of total mortality and cumulative mortality that occurred on each day after initiation of exposure.

Day After Init. of Exp.	Dosage					
	1×10^4		3×10^4		9×10^4	
	% of Total Mortality	Cumulative Mortality	% of Total Mortility	Cumulative Mortility	% of Total Mortility	Cumulative Mortility
1	0	0	0.1	0.1	0.8	0.8
2	1.1	1.1	2.6	2.7	22.3	23.1
3	0.6	1.7	0.7	3.4	6.5	29.6
4	0.4	2.1	0.4	3.8	2.6	32.2
5	0.2	2.3	0.6	4.4	1.9	34.1
6	6.6	8.9	16.4	20.8	7.4	41.5
7	17.9	26.8	20.3	41.1	25.2	66.7
8	24.2	51.0	34.8	75.9	19.8	86.5
9	32.0	83.0	21.3	97.2	9.4	95.5
10	8.3	91.3	2.0	99.2	2.4	98.3
11	5.8	97.1	0.5	99.7	1.0	99.3
12	2.4	99.5	0.3	100.0	0.4	99.7
13	0.5	100.0	0.1	100.1	0.2	99.9

Table 4. Percentages of infection with *Helicosporidium* detected in *Aedes aegypti* larvae.

Day After Initiation of Exposure	Form (L) = Live (D) = Dead	Dosage (Spores/ml)					
		1×10^4		3×10^4		9×10^4	
		#Exam.	%Inf.	#Exam.	%Inf.	#Exam.	%Inf.
2	(L) Larvae	50	38	50	86	50	94
	(D) Larvae	7	28	12	41	50	42
3	(L) Larvae	50	52	50	90	50	92
	(D) Larvae	5	40	7	71	50	44
4	(L) Larvae	50	58	50	84	50	98
	(D) Larvae	2	50	10	100	35	91
5	(L) Larvae	50	84	50	100	50	100
	(L) Pupae	50	44	50	98	50	94
	(D) Larvae	47	48	50	92	50	100
	(D) Pupae	45	54	50	98	38	100
6	(L) Pupae	50	70	50	94	50	100
	(D) Larvae	50	68	50	100	50	100
	(D) Pupae	50	74	50	98	50	100
7	(D) Pupae	50	96	50	100	50	100
	(D) Adults	50	80	50	98	25	96
8	(D) Adults	50	90	50	98	23	96
9	(L) Adults	50	18	50	66	40	60

Table 5. Percentages of infection acquired by exposing 48 hr old *Aedes aegypti* larvae to suspensions of *Helicosporidium* spores stored in demineralized water.

Dose (Spores/ml)	Duration of Storage (weeks)								
	Fresh Spores	1	2	4	6	8	10	12	16
5×10^3	86	90	90	90	88				
1×10^4	100	100	100	98	100	68	80	52	88
2×10^4	100	100	100	100	100				
3×10^4						100	100	90	100
5×10^4	100	100	100	100	100				
7×10^4						100	100	100	100

Table 6. Percentages of infection acquired with *Helicosporidium* spores stored at room temperature in phosphate buffer and in phosphate buffered Polymyxin B.

Dose (Spores/ml)	Baseline % Inf.	Storage Medium			
		0.05 M. Phosphate Buffer		Buffered Polymyxin B	
		10 days	17 days	10 days	17 days
5×10^3	50				
1×10^4	72	4	0	26	0
2.5×10^4	100				
3×10^4		6	0	83	0
5×10^4	100	10	0	68	4
1×10^5		24	2	66	14
3×10^5		28	6	57	26

Table 7. IC_{50}'s of *Helicosporidium* Spores Stored for up to 16 Weeks by Freezing.

Material	Preservation / Storage	Weeks of Storage 4	8	12	16
Spores in intact larvae	Liquid Nitrogen / Liquid Nitrogen	3.5×10^3	1.0×10^4	10^4	2.1×10^3
Spores in intact larvae	Liquid Nitrogen / REVCO (-70°C)	3×10^3	4.5×10^3	2.5×10^3	6.0×10^2
Spore Suspensions in Dimethyl Sulfoxide	Liquid Nitrogen / Liquid Nitrogen	1.2×10^4	5.6×10^3	1.4×10^4	6.4×10^3
Spore Suspensions in Dimethyl Sulfoxide	Liquid Nitrogen / REVCO (-70°C)	1.5×10^4	6.2×10^3	1.1×10^4	5.0×10^3
Spore Suspensions in Glycerine-Egg Yolk	Liquid Nitrogen / Liquid Nitrogen	1.4×10^4	5.4×10^3	1.0×10^4	4.5×10^3
Spore Suspensions in Glycerine-Egg Yolk	Liquid Nitrogen / REVCO (-70°C)	1.4×10^4	9.0×10^2	2.0×10^4	2.0×10^3

IC_{50} of fresh spores = 6×10^3